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for Breast Cancer in Madison County, Alabama

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13. ABSTRACT (Maximum 200 Words) Oakwood College, a private liberal arts institution, in Huntsville, Alabama in collaboration with University of Alabama in Huntsville and University of Birmingham in Huntsville is conducting a research project to study biochemical markers of genotoxicity induced by DDT in Madison County, Alabama. Residents of the town of Triana, predominantly black have been eating fish from a local river contaminated with DDT for nearly 50 years. Recently several epidemiological studies have associated DDT with breast cancer. The purpose of the study is to identify genotoxic biomarkers such as micronuclei induction, cell proliferation and to evaluate the effects of DDT on BRCA1 and BRCA2 gene products and their modulation by the soy protein genistein in rats and exposed humans. A pilot cross-sectional study was conducted among 162 Triana residents, to evaluate all cancers combined in the overall group and breast cancer in women. The occurrence of breast cancer was significantly associated with fish consumed from the DDT contaminated ponds. ($p = 0.0001$). Of the 17 breast cancer cases or their surrogates interviewed, 4 had a family history of breast cancer. Of the remaining 13 breast cancer cases with no family history of breast cancer, 3 cases reported other hormone-related cancers (prostate, pancreas and ovary among immediate family members). Animal experiments were performed with Sprague Dawley (SD) CD rats to determine micronuclei induction and cell proliferating effects of DDT in mammary epithelial cells. Although it appears that DDT may have a promoting effect on cell proliferation, experiments need to be repeated.			
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DOD FINAL REPORT FOR YEAR III

Annual summary report, covering research for the period September 14, 2000 - September 14, 2001

INTRODUCTION

Oakwood College, a private liberal arts institution, in Huntsville, Alabama in collaboration with University of Alabama in Huntsville and University of Birmingham in Huntsville is conducting a research project to study biochemical markers of genotoxicity induced by DDT in Madison County, Alabama. Residents of the town of Triana, predominantly black have been eating fish from a local river contaminated with DDT for nearly 50 years. The study conducted by Center for Disease Control in 1979, confirmed an average total serum levels of 159.4 parts per billion in Triana residents. Recently several epidemiological studies have associated DDT with breast cancer. The purpose of the study is to identify genotoxic biomarkers such as micronuclei induction, cell proliferation and to evaluate the effects of DDT on BRCA1 and BRCA2 gene products and their modulation by the soy protein genistein in rats and exposed humans. The main purpose of the grant however, is to establish the career goals of the PI as an independent breast cancer investigator.

SUMMARY OF RESEARH TRAINING AND ACCOMPLISHMENTS

A database on health effects of DDT on 200 Triana residents has been completed. A pilot cross-sectional study was conducted among 162 Triana residents, to evaluate all cancers combined in the overall group and breast cancer in women and the findings were submitted in the form of an abstract entitled "DDT exposure and breast cancer among Triana residents in Alabama" at the 2000 DOD Era of Hope Meeting.

The occurrence of breast cancer was significantly associated with fish consumed from the DDT contaminated ponds. ($p = 0.0001$). The prevalence odds of breast cancer among those exposed to DDT compared to the non-exposed was 2.4 (95% confidence interval, 0.85-6.5). Of the 17 breast cancer cases or their surrogates interviewed, 4 had a family history of breast cancer. Of the remaining 13 breast cancer cases with no family history of breast cancer, 3 cases reported other hormone-related cancers (prostate, pancreas and ovary among immediate family members). Studies have shown that DDT could impact breast cancer through interactions with the estrogen receptor, suggesting thereby that DDT may have a potential to cause a wide spectrum of hormone related cancers besides breast cancer. Due to the inherent limitations of the cross-sectional study design and the small sample size, a firm interpretation cannot be made.

Animal Experiments:

Animal experiments were performed with Sprague Dawley (SD) CD rats to determine micronuclei induction and cell proliferating effects of DDT in mammary epithelial cells. Female SD rats aged 14 days were obtained from Harlan Sprague-Dawley (Indianapolis,

IN). All animals were acclimated for 7 days prior to use. The animals were housed in cages in an environmentally controlled room (maintained at 22°C and 50% relative humidity) and were fed the diet AIN-76A. Rats were divided into five groups (I-IV). Treatments started at 3 weeks of age. Food and water were available *ad libitum* throughout the experiment. Group I was control. Groups II III and V received DDT (50 mg/Kg body weight, subcutaneously every other day until day 35. Group III received DMBA (40 mg/Kg body weight) by gavage once on day 28 along with DDT treatment. Group IV received DMBA alone. Group V received Genistein in diet (250 mg/Kg diet) along with DDT and DMBA.

Design:

Group	Treatment	Dose	Day given
Group I	Control, sesame oil	-	21, 23, 25, 27, 29, 31, 32, 35
Group II	DDT	(50 mg/Kg body weight)	21, 23, 25, 27, 29, 31, 32, 35
Group III	DDT +	(50 mg/Kg body weight)	21, 23, 25, 27, 29, 31, 32, 35
Group IV	DMBA	(40 mg/Kg body weight)	28
Group V	DMBA DDT + DMBA + genistein	(40 mg/Kg body weight) (50 mg/Kg body weight) (40 mg/Kg body weight) (250 mg/Kg diet)	28 21, 23, 25, 27, 29, 31, 32, 35 28 <i>ad libitum</i> , mixed in diet

Body weight of animals was monitored daily. The rats were killed on day 14. One hour before being killed they were administered 5-bromo-2'-deoxyuridine (BrdU) (100 mg/Kg) was given intraperitoneally. Rats were dissected and mammary gland was removed. Breast tissue was separated and cells were scraped with a blunt scalpel. The tissue was mounted on a slide. Before completely drying, the tissue was fixed in methanol. Cells were stained with cytokeratin antibody to label mammary cells and counterstained with DAPI to identify the micronuclei. Some of the cells were stained with BrdU to study cell proliferation. Due to difficulty in scoring the cells, the method was modified. Mammary tissue was frozen and sections were made to study micronuclei and cell proliferation. (See photographs VI and VII)

Processing mammary tissue for immunohistochemistry

Mammary glands were dissected and tissue was placed in cryomolds containing O.C.T embedding medium. Samples were flash frozen by immersing in a metal cup filled with 2-methyl butane pre-chilled in a larger metal cup containing liquid nitrogen. Frozen blocks were wrapped in aluminum foil and stored in an -80°C freezer and were latter sectioned on a cryostat. Five micron sections were collected on (+) charge slides and were fixed just before drying in Methanol/acetone (3:1) for 30 minutes on ice. Samples were then briefly rinsed in PBS and processed for immunofluorescence.

BrdU labeling and micronucleus assay

Replicating cells were studied using BrdU incorporation. BrdU labeling was conducted by denaturing the cells in 0.07N NaOH followed by neutralization with phosphate-buffered saline (PBS). The slides were then incubated with an anti-BrdU antibody, diluted in 0.5%Tween-20 in PBS, in a humidified chamber for 30 min. The antibody was then detected by incubation with Texas Red-conjugated goat anti-mouse IgG (10 µg/ml) in a humidified chamber for 30 min. After washing the slides in PBS, the DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1µg/ml) in antifade mounting medium. The cells were analyzed at the UAB Imaging Facility. Microscope utilized was Leitz Orthoplan with epifluorescence and Hoffman Modulation Contrast optics equipped with a Photometrics CH250 liquid cooled CCD, high resolution, monochromatic camera. For visualization of green, red, and blue fluorochromes, 83000 Pinkel filter set from Chroma Technology was utilized. Image acquisition software was IPLab Spectrum from Scanalytics.

Human studies have been initiated. Gene expression studies have been conducted in year I with MCF -10A Human Mammary Epithelial cell line and BRCA1 and BRCA gene products have been identified. Currently Studies are being initiated with blood samples from Triana residents using the following protocol. The study population includes residents of Triana who were exposed to DDT and. Initially, 20 volunteers will donate blood for examination. Controls will be those subjects that were not exposed to DDT. Blood was drawn by venipuncture from three residents. Density gradient centrifugation (200g for 15 minutes at 20°C) was used to separate plasma, leukocyte-rich plasma (buffy coat) and erythrocytes. Erythrocytes are stored at -70°C. Plasma was further centrifuged (2500g for 15 minutes at 20 °C) to pellet the platelets. The resulting supernatant, which was cell-free, platelet-free plasma (CFPF), was stored at -70°C. Leucocyte-rich plasma (buffy coat) was further centrifuged to separate lymphocytes. The purified lymphocyte fractions was used to extract total RNA. RT-PCR will be used to amplify the BRCA1 and BRCA2 genes. Agarose gel electrophoresis will be used to determine if the presence of the mutations BRCA1 C insertion 282, BRCA1 AG deletion 235 or BRCA2 T deletion 417.

Due to technical difficulties, tasks were not accomplished as scheduled. An extension at no cost is granted until September 2002

TASK I completed

Generated a database on health effects of 200 Triana residents. Statistical analysis was done and an abstract was submitted entitled "DDT exposure and breast cancer among Triana residents in Alabama"

TASK II Confirm the genotoxicity of DDT in exposed human breast nipple aspirates and blood samples

Perform modified micronucleus assay using an antikinetochore antibody (6-24)

Isolation of lymphocytes from exposed Triana population

- Protocol was developed for conducting the chromosome specific probe assay and BrdU labeling of DNA synthesis assay with blood lymphocytes.
- Detect aneuploidy by the chromosome-specific DNA probe (6-30)
- Mammary epithelial cells are processed to study this assay. Probes have not been used on the cells yet.

TASK III Develop assays for other possible mechanisms of DDT carcinogenicity (months 6-30)

- Induction of altered gene expression, genotoxicity by selected breast cancer genes will be evaluated (6-30)
RT-PCR studies on lymphocytes for expression of BRCA 1 and BRCA 2 are being conducted. No data available yet. Studies were conducted with MCF-10A Human Mammary Epithelial cells.
- RNA has been extracted from three blood samples from Triana residents. RT-PCR studies have been initiated.

TASK IV Genotoxic assays performed in vivo in Sprague-Dawley rats.

- Perform micronucleus assay, chromosome-specific DNA probe assay, DNA synthesis assay with or without soy proteins, isothiocyanates and organ sulfur compounds from garlic
- Experiments have been conducted in vivo in Sprague-Dawley rats for micronuclei induction and DNA synthesis assay with soy protein genistein.

KEY RESEARCH ACCOMPLISHMENTS

September 1999-September 2000

- A database on health effects of DDT on 200 Triana residents has been completed.
- A pilot cross-sectional study was conducted among 162 Triana residents, to evaluate all cancers combined in the overall group and breast cancer in women and the findings were submitted in the form of an abstract entitled "DDT exposure and breast cancer among Triana residents in Alabama"
- A new technique was developed under the expertise of a UAB pathologist to isolate mammary epithelial cells.
- The cells were then stained with cytokeratin antibody to identify mammary cells and then counterstained with DAPI to study the micronuclei. Some of the cells were

stained with BrdU to study cell proliferation. The cells were analyzed at the UAB Imaging Facility.

September 2000-September 2001

- Animal studies have been conducted. Photographs from various treatment groups have been enclosed. See Photographs I-V. Although it appears that DDT may have a promoting effect on cell proliferation, experiments need to be repeated. A method of Quantitating fluorescence for various treatment groups is developed .
- To make scoring more accurate, tissue was frozen and sections were prepared for immunohistochemistry. This revealed the entire glandular structure and changes in the nucleus. See photographs VI and VII
- Human studies have been initiated. Total RNA has been extracted from three women. RT-PCR studies have been initiated.

- **REPORTABLE OUTCOMES:**

September 1999-September 2000

- The findings of a pilot cross-sectional study was conducted, to evaluate all cancers combined in the overall group and breast cancer in women and the findings were submitted in the form of an abstract entitled "DDT exposure and breast cancer among Triana residents in Alabama" at the 2000 DOD Era of Hope Meeting.

September 2000 - September 2001

- Initial animal studies indicate that DDT may have a promoting effect on cell proliferation when used with the mammary carcinogen DMBA
- A grant entitled "Environmental Justice for the community of Triana -Exposed to DDT" is pending with the Alabama State.
- Received the 2002 AACR-HBCU Faculty Scholar in Cancer Research Award by the American Association for Cancer Research.
- A manuscript entitled "DDT exposure and breast cancer among Triana residents in Alabama" is in preparation

LEGENDS FOR PHOTOGRAPHS

I - V Rat breast tissue exposed to DDT, labeled with BrdU and counter-stained with DAPI to examine cell proliferation

I Control

II DDT exposed cells

III DMBA induced cell proliferation

IV DDT and DMBA exposed cells

V Cells treated with DDT, DMBA and genistein

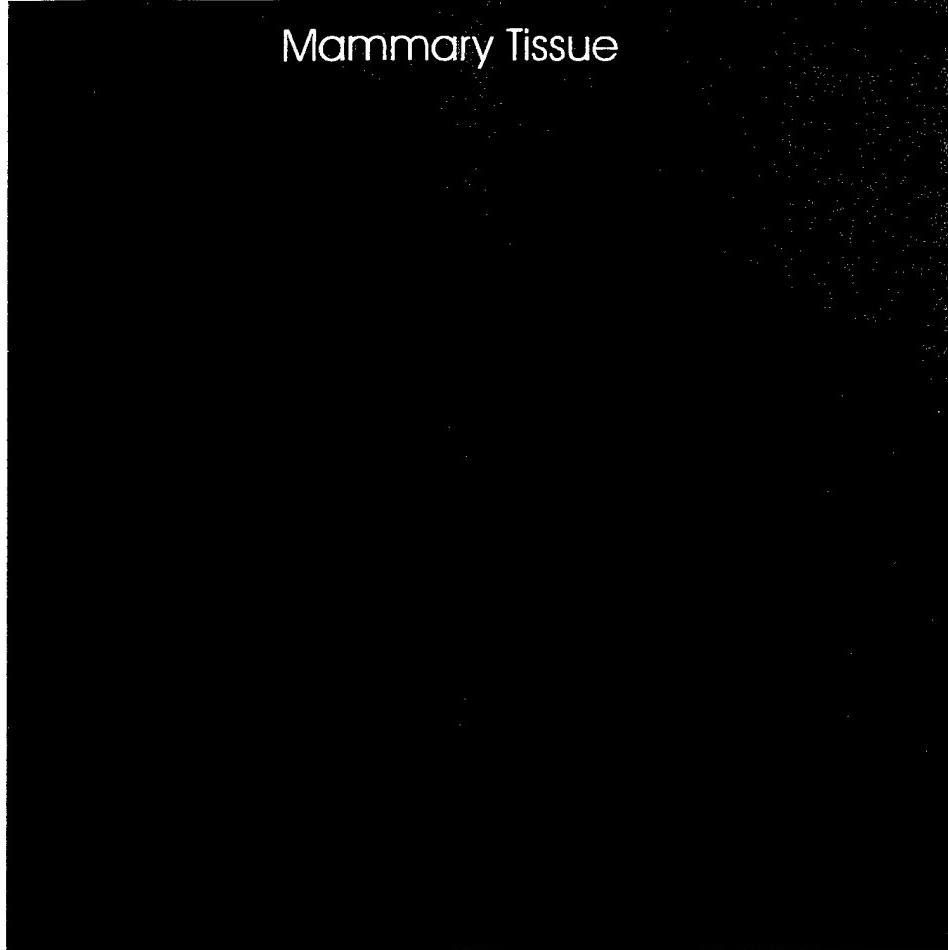
VI & VII Representative examples of paraffin sections of rat prostate gland (X40) and (X100)

I

Control

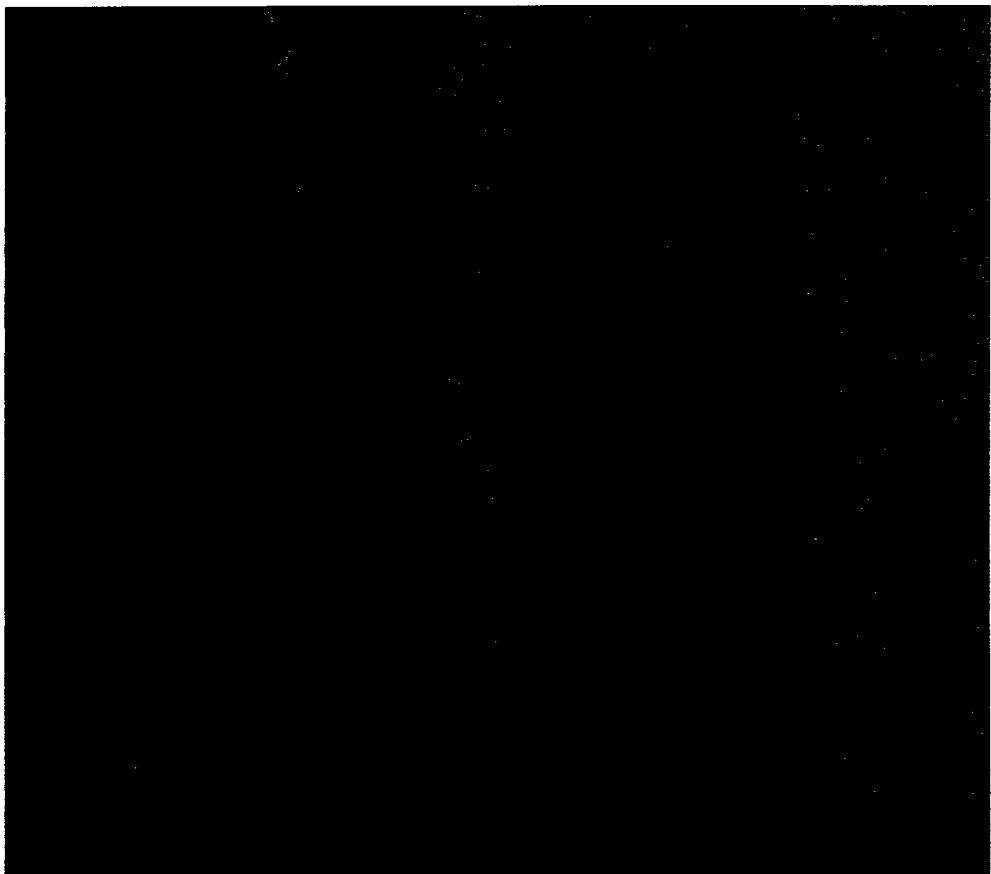
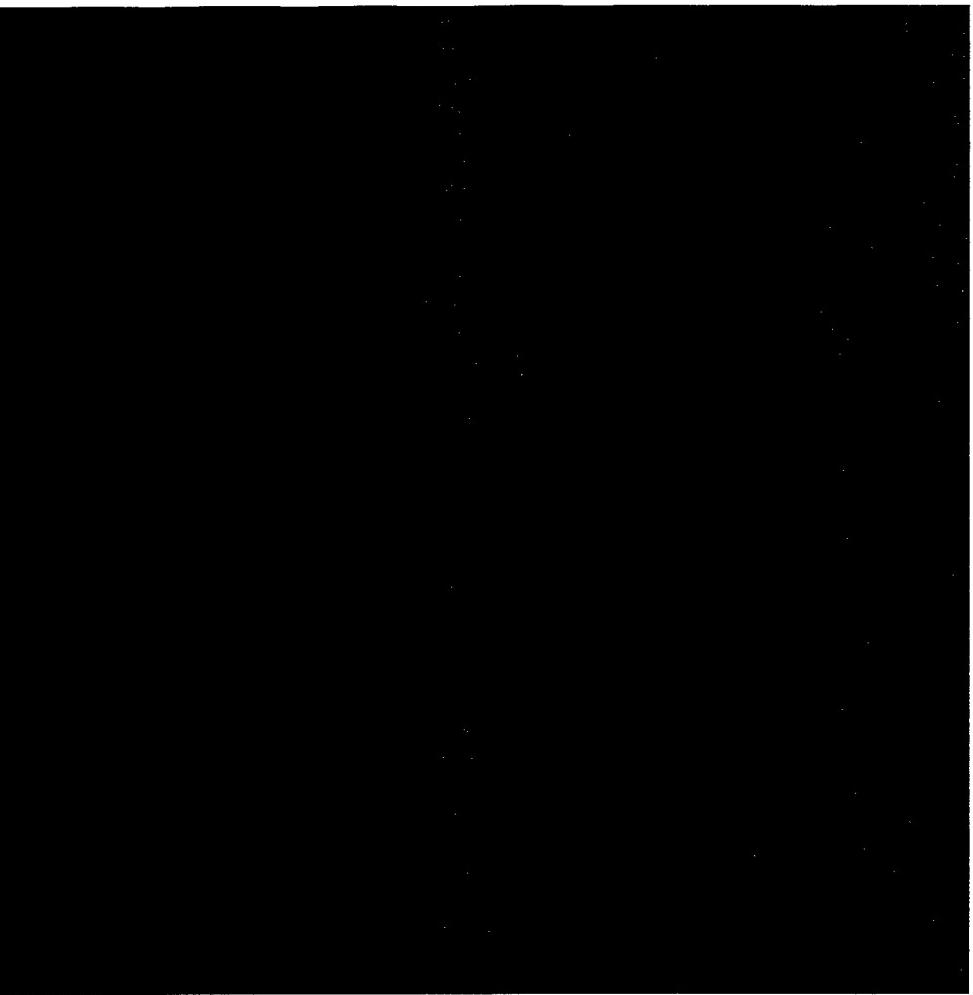
DDT

Mammary Tissue



DMBA - BRDU incorporation

DDT + DMBA



Genistein + DDT + DMBA



